Melanin concentrating hormone in peripheral circulation in the human

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AUTHORS: J. Naufahu, F. Alzaid[1], M. Fiuza Brito[2], B. Doslikova[3], T. Valencia[4], A. Cunliffe[5], J.F. Murray[6]

ADDRESS: Faculty of Science and Technology, University of Westminster, 115 New Cavendish Street, London, W1W 6UW, United Kingdom

1. Current address: Sorbonne Universités, Université Pierre et Marie-Curie; INSERM, Centre de Recherches des Cordeliers, UMRS 1138, Paris, 75006, France

2. Current address: Royal Brompton & Harefield NHS Foundation Trust, Sydney Street, London, SW3 6NP, United Kingdom

3. Current address: Department of Pharmacology, University of Alberta, 9-36 MSB, T6G 2H7, Canada

4. Current address: Regulus Therapeutics Inc., 10614 Science Center Drive San Diego, CA 92121, United States of America

5. Current address: Department of Applied Sciences, London South Bank University, 103 Borough Road, London, SE1 0AA, United Kingdom

6. Current address: Centre for Integrative Physiology, Hugh Robson Building, George Square, University of Edinburgh, Edinburgh, EH8 9XD, United Kingdom

CORRESPONDING AUTHOR and REQUESTS FOR REPRINTS:

Joanne F. Murray

Edinburgh Medical School: Biomedical Sciences

Centre for Integrative Physiology

Hugh Robson Building
George Square
University of Edinburgh
Edinburgh, EH8 9XD
United Kingdom
jmurra19@exseed.ed.ac.uk
T: +44 (0)131 651 1711

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ABSTRACT

Melanin concentrating hormone (MCH) is a hypothalamic neuropeptide with a well-characterised role in energy homeostasis and emergent roles in diverse physiologic functions such as arousal, mood and reproduction. Work to date has predominantly focused on its hypothalamic functions using animal models however little attention has been paid to its role in circulation in humans. The aims of this study were to a) develop a radioimmunoassay for the detection of MCH in human plasma; b) establish reference ranges for circulating MCH; and c) characterize the pattern of expression of circulating MCH in humans. A sensitive and specific RIA was developed and cross-validated by RP-HPLC and MS. The effective range was 19.5–1248 pg MCH/ml. Blood samples from 231 subjects were taken to establish a reference range of 19.5–55.4 pg/ml for fasting MCH concentrations. There were no significant differences between male and female fasting MCH concentrations however there were correlations between MCH concentrations and BMI in males and females with excess fat ($p<0.001$ and $p=0.020$) and between MCH concentrations and fat mass in females with excess fat ($p=0.038$). Plasma MCH concentrations rose significantly after feeding in a group of older individuals ($n=50$, males $p=0.006$, females $p=0.023$). There were no robust significant correlations between fasting or post-prandial MCH and resting metabolic rate, plasma glucose, insulin or leptin concentrations although there were correlations between circulating MCH and leptin concentrations in older individuals ($p=0.029$). These results indicate that the role of circulating MCH may not be reflective of its regulatory hypothalamic role.

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INTRODUCTION

Melanin concentrating hormone (MCH), is an orexigenic neuropeptide; rodent studies indicate it has multiple and diverse physiologic functions including a key role in the central control of energy metabolism. Intracerebroventricular (ICV) administration of MCH results in hyperphagia and increased adiposity (Qu *et al.,* 1996; Gomori *et al.,* 2002; Santollo and Eckel, 2008), whilst decreased availability of hypothalamic MCH results in hyper- or hypophagia accompanied by reduced body weight and fat mass depending on whether a pharmacological or genetic model is used (Marsh *et al.,* 2002; Segal-Lieberman *et al.,* 2003; Mashiko *et al.,* 2005). Ablation of functional MCH results in increased energy expenditure via increased metabolic rate, increased locomotor activity, or both (Shimada *et al.,* 1998; Segal-Lieberman *et al.,* 2003). MCH is expressed in the central nervous system (CNS), primarily in the rostral zona incerta/incerto-hypothalamic and the lateral hypothalamic areas (Bittencourt *et al.,* 1992; Sita *et al.,* 2007; Bittencourt, 2011). Prepro-MCH mRNA and MCH have also been reported in rodent and human peripheral tissue (Hervieu and Nahon, 1995; Verlaet *et al.,* 2002; Sandig *et al.,* 2007). Circulating MCH has been detected in both rodents (Bradley *et al.,* 2000; Stricker-Krongrad *et al.,* 2001; Sun *et al.,* 2004) and humans (Gavrila *et al.,* 2005; Schmidt *et al*., 2015) however there has been published debate concerning the validity of the detection methods used in the earlier human study (Mantzoros, 2005; Waters and Krause 2005). Both central and peripherally-derived MCH are implicated in glucose homeostasis (Ludwig *et al.,* 2001; Pereira-da-Silva *et al.,* 2005; Bjursell *et al.,* 2006) and there is evidence of local production of MCH in the endocrine pancreas in rodents and humans (Pissios *et al.,* 2007). However the physiological role of circulating MCH remains largely unexplored at present.

The overall aim of these studies was to determine whether circulating concentrations of MCH are related to body weight regulation and metabolism by developing and validating a competitive RIA for the detection of MCH in human plasma, and conducting a cross-sectional study in order to establish reference ranges for circulating MCH. Two intervention studies were also conducted to investigate whether circulating MCH concentrations were acutely responsive to food stimuli, furthermore plasma MCH concentrations in both the fasted and fed states were examined in association with circulating glucose, insulin and leptin concentrations. Additionally, associations between circulating MCH and resting metabolic rate (RMR) were investigated.

MATERIALS AND METHODS

MCH RIA development and validation

Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) and Mass Spectrometry (MS)

RP-HPLC was conducted using a modified version of a previously described method (Maulon-Ferraille *et al.,* 2002). The optimum dilution for detection of MCH in plasma was 1:9 plasma: 0.1 N HCl (v:v). The mixture was centrifuged at 4°C and 1000 rpm for 10 minutes and the supernatant analyzed by HPLC in a RP column (C18 Phenomenex, UK) with a gradient of 20–60% (0.1% trifluoroacetic acid in HPLC water: acetonitrile) for 60 minutes at a flow rate of 0.5 ml/min. Purified MCH was serially diluted and treated similarly for comparison. MCH was detected using UV absorbance at 230 nm. Protein fragments obtained by RP-HPLC were subject to MS for determination of analyte mass. MS was performed by single quadropole mass spectrometric detector (Dionex MSQ Plus, Dionex Corp., Massachusetts, USA) and MS data analysed using Chromeleon LC/MS software (Dionex Corp., Massachusetts, USA).

Blood from the same individual was collected in different vacutainers (lithium heparin, silica+gel, fluoride oxalate, EDTA and sodium citrate) to establish if there was any effect on the detection of MCH by RP-HPLC. To determine the lability of MCH in plasma, a separate blood sample was subjected to the following conditions: room temperature 1 hour; 4°C 1 hour; room temperature overnight; 4°C overnight; -20°C overnight; and -20°C before being thawed, refrozen and thawed again. The samples were processed as described above and compared with a freshly prepared sample.

RIA for MCH

A double antibody RIA for MCH was developed using commercially available reagents; that is, MCH antibody (M8440: Sigma-Aldrich, UK), radiolabelled MCH (^{125}I-MCH; NEX373010UC: PerkinElmer Inc., USA) and anti-rabbit SacCel (AA-SAC1: IDS Ltd., UK). Phosphate buffered saline with 1% bovine serum albumin (A3294: Sigma-Aldrich, UK) was used throughout. Day 1: MCH antibody (1:30,000 in 100 μl) with normal rabbit serum (1:300) was added to diluted standards and unknowns and left at 4°C. Day 2: ^{125}I-MCH (10,000 cpm/100 μl) diluted in buffer supplemented with EDTA (0.025 M) was added to each tube and left at 4°C. Day 3: SacCel (solid phase anti-rabbit IgG coated cellulose suspension: IDS Ltd., Boldon, UK) was added following the manufacturer's instructions; that is, 0.1 ml SacCel were added to each tube (except total counts), left for 30 minutes at room temperature and then 1 ml deionized water was added before all tubes were centrifuged at 1000 rpm and 4°C for 10 min. The supernatant was aspirated and the resultant pellet was counted for 1 minute on a gamma counter. Data were analysed using AssayZap (Biosoft, Cambridge, UK).

To determine possible cross-reactivity, a series of dilution curves (range 0.1 pg–0.1 mg) of biomolecules reported to have a competitive or agonistic relationship with MCH were treated as unknowns in the MCH assay. Biomolecules tested were: human atrial natriuretic peptide (ANP; A1663: Sigma-Aldrich, UK) (Hervieu *et al.,* 1996); human α-MSH (H1075: Bachem, Switzerland) (Barber *et al.,* 1987; Ludwig *et al.,* 1998); human ACTH (H1160: Bachem, Switzerland) (Baker *et al.,* 1985); and neuropeptide–E-I-MCH (NEI-MCH; H4714: Bachem, Switzerland) (Maulon-Ferraille *et al.,* 2002).

Comparison between RP-HPLC and RIA

Plasma samples collected in EDTA tubes were diluted with either 0.1 N HCl or buffer with EDTA (1:9 dilution) with/without purified MCH and subjected to RP-HPLC. Fractions were collected at 1 minute intervals and the aliquots analysed for MCH by RIA.

<u>Circulating MCH</u>

Subjects

The experiments involving human subjects were approved by the University of

Westminster's Ethics Sub-Committee. Each subject gave full informed consent. Fasting blood samples were taken from all subjects between 8 and 11.30 am.

Cross-sectional study: Fasting venous blood samples were collected from 135 females and 96 males. Weight to the nearest 0.1 kg, height to the nearest 0.1 cm, waist and hip circumference were measured. Total fat and lean body mass were measured by air displacement plethysmography (BodPod: Body Composition Tracking System, Version 4.1; Life Measurement Instruments, Concord, CA, USA). All venous blood samples were collected in EDTA vacutainers and plasma recovered after centrifugation. Plasma was stored at -20˚C until MCH concentrations were determined by RIA (intra- and inter-assay CVs were 2.4% and 3.7%, respectively).

Intervention studies: Two cohorts were recruited. Cohort A) 18–30 years: 21 females and 11 males. The inclusion criteria for females were: pre-menopausal (however the stage of the menstrual cycle was not recorded); non-hormonal contraceptive using; and a body mass index (BMI) of ≤24.9. The inclusion criterion for males was BMI of ≤24.9. Cohort B) Over 40 years: a) lean individuals (11 females and 11 males) and b) those with excess body fat (13 females and 15 males). Lean (L) was defined as <31 % body fat in females and <21 % body fat in males. Excess body fat (E) was defined as ≥31 % body fat in females and ≥21 % body fat in males (ACSM, 1996). The inclusion criterion for both males and females was that they should be over 40 years of age. In both cohorts, those on medication(s) for chronic illness or known to cause hypo- or hyperglycaemia or affect metabolic rate and females who were pregnant, lactating or recently lactating were excluded.

Protocol. Subjects arrived after an overnight fast, anthropometric, body composition and resting metabolic rate (RMR) (Deltatrac II Metabolic Monitor, Datex Instrumentarium Corp., Helsinki, Finland) measurements were made. Fasting venous and fingerprick blood samples were obtained before subjects were fed a controlled meal of mixed macronutrient content (388 k/cal females; 510 k/cal males). Eight fingerprick samples were obtained at 15 minute intervals and 3 venous blood samples at 30, 60 and 120 minutes post meal. Plasma was recovered from the venous blood samples and stored at -20˚C until assayed for MCH, leptin (HL-81HK, Millipore, USA; intra-assay CV: 8.3% at 4.9 ng/ml; 3.4% at 25.6 ng/ml) and insulin (DSL-1600, Diagnostic Systems Inc., USA; intra-assay CV: 8.3% at 4.8 µIU/ml; 6.4% at 54.6 µIU/ml). All samples for each cohort were assayed for each hormone in a single assay. The fingerprick blood samples were immediately analysed for blood glucose concentrations using the Hemocue Glucose 201+ Analyser (Hemocue AB, Sweden; intra-assay CV <1.8%).

Data and Statistical Analyses

RMR: The group was sub-divided based on percentage of “Standard BMR” (Fleisch, 1951). A BMR of ±10% Standard is considered normal (McArdle *et al.,* 2001). The groups were Low (L)= ≤89.9% Standard BMR; Normal (N)=within 10% of Standard BMR; High (H)= ≥110% Standard BMR. RMR has been used synonymously with BMR since the only condition specific to BMR that was not met was that subjects did not sleep at the facility overnight.

Body composition: subjects were sub-divided into four groups based on the American College of Sports Medicine’s body fat percentage cut-off points: male lean

(ML)=body fat % <21%; male excess fat (ME)=body fat % ≥21%; female lean (FL)=body fat % <31%; female excess fat (FE)=body fat % ≥31% (ACSM, 1996). Subjects were sub-divided by body composition after data collection for the cross-sectional and both intervention studies.

Inter-gender differences between anthropometric characteristics and circulating hormone concentrations were established by independent samples t-tests. A one-way between groups ANOVA with Tukey's Multiple Comparison test was conducted to determine whether there was an effect of body composition on plasma MCH concentrations. Associations between fasting plasma MCH concentrations and body composition parameters were determined by Pearson product-moment correlational analysis. Differences in pre- and post-prandial circulating hormone concentrations were assessed by paired samples t-tests. Leptin concentrations were not normally distributed and so the data were transformed using the square root before the analyses. Comparisons between circulating hormone concentrations at the four sampling time-points were assessed by repeated measures design ANOVA. When analyzing the AUC data, only individuals with data from all four blood samples were included in the analyses and hence the lower 'n' values. Data were analysed using the Statistical Package for the Social Sciences (SPSS version 16.0 for Windows; Chicago, IL, US) or Prism (Prism 5 for Mac OS X; GraphPad Software, Inc.). Statistical significance was set at $p<0.05$.

RESULTS

RIA development and validation

The gold standard method for the detection of MCH is RP-HPLC and MS hence this method was used to demonstrate that MCH is present in plasma. Using RP-HPLC, the retention time for purified MCH was between 21 and 28 minutes (Figure 1a). It was predicted that product ions of m/z 796 and 2 of m/z 1194 would be generated specifically for MCH and these were detected at the corresponding elution times when either purified MCH or human plasma samples were analysed by MS (Figure 1b). MCH was only detected by RP-HPLC and MS in samples collected in the lithium heparin, silica+gel and EDTA vacutainers. No effect of storage under the conditions described could be detected when compared to freshly prepared samples measured by RP-HPLC and MS (data not shown). Purified MCH, plasma and buffer alone were each separately fractionated by RP-HPLC and eluates collected at one minute intervals. Immunoreactive MCH, as determined by RIA, was detected in eluates collected between 19 and 28 minutes for purified MCH and eluates collected between 18 and 24 minutes for plasma (Figure 1c).

An effective range of 19.5–1248 pg MCH/ml was established and 19.5 pg/ml was taken as the level of detection. Serial dilutions of ANP, α-MSH and ACTH failed to displace the MCH antibody demonstrating the specificity of the radioimmunoassay. Only supraphysiological concentrations of NEI-MCH showed any potential for cross-reactivity with the MCH antibody (at concentrations 100x greater than MCH: data not shown). Standard curves diluted in buffer or plasma (with unknown initial concentrations of MCH: data not shown) were parallel.

<u>Circulating MCH in humans</u>

Cross-sectional study: Demographic and anthropometric measurements of 231 subjects are presented in Table 1. Fasting plasma MCH concentrations were detected in the range 19.5–70.4 pg/ml with the exception of one subject who had fasting MCH concentrations in excess of 150 pg/ml (this outlier was not included in Table 1 or in the statistical analyses). Within this assay 95% of the sample population would be expected to have fasting MCH concentrations between 19.5 and 55.4 pg/ml. There were no significant differences in mean fasting plasma MCH concentrations between males and females. When the sample population was grouped by gender and BMI, there were no significant differences in plasma MCH concentrations between the groups (Figure 2a) except between males with a BMI <20 compared with those with a BMI >30 (p<0.0473, ANOVA with Tukey's Multiple Comparison test). Fasting plasma MCH concentrations were not significantly correlated with percent fat mass, percent lean mass, height, weight or age. There were however significant correlations between fasting plasma MCH concentrations and: a) both body fat mass weight (kg) and BMI in females with excess fat (≥31% body fat) (n=41, r=-0.326, p=0.038; n=39, r=-0.372, p=0.020, respectively); b) male BMI (n=96, r=0.230, p=0.030); and c) BMI in males with excess fat **(**≥21% body fat) (n=44, r=0.513, p<0.001: Figure 2b). Note that in females the correlations were inverse whilst in males correlations were positive.

Intervention studies: Demographic, anthropometric and fasting MCH measurements are presented in Tables 2 and 3 for Cohorts A and B, respectively. There were differences in the post-prandial plasma MCH concentrations between Cohort A (n=32) and Cohort B (n=50). In Cohort A, there were no differences in plasma MCH concentrations at any of the four time-points (p=0.772; Figure 3). In Cohort B, plasma MCH concentrations increased after eating (females p=0.023, males p=0.006; Figure 4).

There were no differences in circulating concentrations of glucose or insulin between males and females and no effect of % body fat in either Cohort A or Cohort B. In Cohort A, although there were no correlations with mean plasma MCH concentrations or the MCH area under the curve (AUC) and the glucose AUC, both the mean plasma MCH concentrations and the MCH AUC were correlated with the insulin AUC in all individuals (that is, both females and males) with excess fat only (that is, ≥31% body fat in females and ≥21% in males) (n=6, r=0.907, p=0.013 and n=6, r=0.932, p=0.007, respectively). In Cohort B, there were no significant associations between mean plasma MCH concentrations or the MCH AUC and the glucose and insulin AUCs.

Mean circulating leptin concentrations were greater in individuals with excess fat compared to lean individuals (Cohort A, p=0.025; Cohort B, p>0.001) and women have higher concentrations than males (Cohort A, p>0.001; Cohort B, p>0.001): Figures 3 and 4). In both cohorts in females, circulating leptin concentrations had decreased 1 hour post-prandial (Cohort A: p=0.012; Cohort B: p=0.037). In males, circulating leptin concentrations did not decrease significantly until 2 hours post-prandial in Cohort B only (p=0.028). In Cohort A, there were no significant correlations between plasma MCH and leptin at any time-point or the MCH and leptin AUCs. In Cohort B there were three significant correlations between plasma MCH and leptin concentrations. There were negative correlations between plasma MCH concentrations and leptin concentrations in fasted males with excess fat (that is, ≥21% body fat) (n=9, r=-0.672, p=0.047) and at 30 minutes post-prandial in females with excess fat (that is, ≥31% body fat) (n=8, r=-0.757, p=0.030). By contrast, there was a positive correlation between mean plasma MCH concentrations and mean plasma leptin concentrations in lean males (that is, <21% body fat) (n=11, r=0.654, p=0.029). The MCH AUC was not significantly correlated with the leptin AUC.

In both cohorts, there were no significant correlations between fasted or mean post-prandial plasma MCH concentrations and RMR (for values, Tables 2 and 3) in either males or females or when categorized by adiposity.

DISCUSSION

A sensitive and specific RIA for the quantifiable measurement of MCH in human plasma has been successfully developed. To confirm that MCH is detectable and measurable in human plasma cross-validation was performed by RP-HPLC and MS. A peak was detected between 21 and 28 minutes when plasma was run through the HPLC column which corresponds to the elution time of purified MCH. Additionally, when human plasma was subject to MS, product ions of identical mass to those generated by purified MCH were observed. Immunoreactive MCH was detected by RIA only in the eluates collected between 18 and 28 minutes of either purified MCH or human plasma. In the RIA, the only molecule assessed showing evidence of cross-reactivity was NEI-MCH, though only at supra-physiological concentrations. Currently there is little evidence to suggest that NEI-MCH circulates therefore at physiological concentrations this assay is specific for MCH. Furthermore, parallelism of the dilution curves of plasma to the standard curve confirmed that other plasma components have no adverse effects on the curve. Plasma MCH retained stability under various conditions including freeze-thaw cycles and being left at room temperature overnight. MCH was only detected in plasma collected in lithium heparin or EDTA vacutainers or in serum tubes containing a clotting agent. Other anti-coagulants interfered with detection. These results indicate that collection methods for plasma MCH should be standardized, though variability in storage conditions is not detrimental.

Using the RIA described herein, repeatable measures of the relative concentrations of MCH in circulation have been obtained. The range of values obtained do vary significantly however from the two other studies published to date (Gavrila *et al*., 2005; Schmidt *et al*., 2015). The two other research groups used two different assays from the same commercial supplier and it is not known if the assays utilize the same antibody. Neither group appeared to validate the assay they have used within their own laboratories and there is little information supplied by the company to suggest that the assays have been validated by the company itself. Whilst it is not uncommon, as Schmidt and colleagues noted in their discussion on the differences in measurements for MCH in their studies and Gavrila and colleagues', for there to be a wide range of baseline values reported depending on the method of assaying (for example, B-type natriuretic peptide [as reviewed by Fischer *et al*., 2001] and oxytocin [as reviewed by Leng and Ludwig, 2016], the paucity of validation data available for the two commercial assays does preclude direct comparisons being made between their findings and those described herein.

Fasting blood samples from 135 females and 96 males were obtained to establish a reference range. Subjects were recruited from a range of ethnicities, ages and phenotypes. The mean fasting plasma MCH concentration was 36.7±9.3 pg/ml and

95% of the population would be expected to have plasma MCH concentrations between 19.4-55.4 pg/ml. In rodents, increased availability of hypothalamic MCH is associated with adiposity (Ludwig *et al.,* 2001; Gomori *et al.,* 2002) whilst decreased availability is associated with leanness (Marsh *et al.,* 2002; Kowalski *et al.,* 2004), therefore it was hypothesized that circulating MCH concentrations would also be aligned to fat mass in humans. Whilst there were no associations between percent fat mass, percent lean mass, age, height or weight: there were significant correlations between circulating MCH concentrations and BMI in males and females with excess fat (that is, ≥31% body fat in females and ≥21% in males). There was also a correlation between circulating MCH concentrations and body fat weight (kg) in females with excess fat. These results are curious since the correlations between BMI and MCH were inverse for women and positive for men. Hence it appears there may be some gender and age related regulation which differs in the presence of adiposity. In young males MCH may be indexed to leanness rather than adiposity since there was a positive correlation between BMI and lean body mass (kg) in the younger cohort. The two major peripheral adiposity signals; leptin and insulin are processed differently in males and females, female brains being more sensitive to leptin and male brains being more sensitive to insulin. Leptin correlates better with total body fat in females and insulin correlates better with total body fat in males (Clegg *et al.,* 2003; Woods *et al.,* 2003). It could be that MCH also displays a sexually dimorphic sensitivity: whether or not fat interferes with MCH signalling either directly or indirectly via leptin resistance or some other perturbation of the system is not currently known.

There were no differences between male and female fasting circulating MCH concentrations. In this respect, our results agree with those of Gavrila and colleagues (Gavrila *et al.,* 2005). Age-related changes in body composition did not appear to impact on circulating MCH concentrations since there were no differences in circulating MCH concentrations between groups when all subjects in the cross-sectional study were categorized by gender, age (≤30 years, 31-39 years and ≥40 years) and % body fat (male lean=<21 %; male excess fat=≥ 21%; female lean=<31 %; female excess fat=≥31%). Nor was there a significant correlation between age and absolute circulating MCH concentrations. However in the intervention studies an effect of age was observed, both in fasting MCH concentrations which were greater in the younger group compared to the older group and in the post-prandial response (compare Figures 3 and 4).

In the older group circulating MCH concentrations rose significantly during the 2 hour post-prandial sampling period in males and females, and in both lean individuals and those with excess fat. In the younger group, post-prandial circulating MCH concentrations did not change significantly. In both groups, post-ingestive circulating leptin concentrations declined significantly. Whether or not this was related to the meal, MCH concentrations or the morning nadir of leptin requires qualification (Sinha *et al.,* 1996). In the lipostatic model of energy homeostasis (for review see Woods, 2005), leptin inhibits the anabolic pathway through which MCH operates. Leptin and MCH may also interact in the periphery; for example, the MCH receptor has been detected on rodent adipocytes (Bradley *et al.,* 2000). Whilst there is ample evidence that MCH and leptin can both inhibit and stimulate each other (Huang *et al.,* 1999; Bradley *et al.,* 2000; Kokkotou *et al.,* 2001)), few studies have attempted to evaluate the association between circulating MCH and leptin. Except for in 3 small sub-groups (within Cohort B) there were no consistent significant associations between circulating MCH and leptin concentrations: in this respect our results broadly concur with Gavrila and colleagues (2005) who found no associations with serum leptin concentrations in a younger population (17±1.7 yrs). Leptin action is altered with aging and is characterized by increased adiposity and the development of leptin resistance: it is not known which precedes which (Carrascosa *et al.,* 2009). In the older individuals, it may be that the differential direction of the plasma MCH/plasma leptin relationship between those with excess fat and lean phenotypes (negative *versus* positive) is symptomatic of disruption between MCH and leptin signaling, in this context it is possible that MCH may be responding to some leptin resistant state. Overall there was a trend for circulating MCH and leptin concentrations to be inversely correlated which, whilst non-significant, was consistent. This would be expected if the inhibitory effect of hypothalamic leptin on hypothalamic MCH is reflected in the periphery.

MCH has been shown to stimulate insulin release from beta cells *in vitro* and it has been suggested that MCH may be necessary for normal β-cell function (Pissios *et al.,* 2007). Whether or not MCH acts in a paracrine or autocrine manner within the pancreas or is released into the circulation is not known. If MCH is active at the level of the endocrine pancreas it was hypothesised that the post-prandial insulin response might be related to the post-prandial MCH response and could be altered in the presence of insulin resistance. Whilst there was a gender difference in the magnitude of the AUC insulin, which did not appear to be related to adiposity, our results indicate that there were no robust associations between the MCH AUC or mean circulating MCH concentrations and the glucose or insulin AUCs. However in the younger cohort, there was a significant positive relationship between the respective AUCs for insulin and MCH, and with the insulin AUC and mean circulating MCH concentrations but only in individuals with excess fat. Whilst in this group it would seem that the MCH and insulin response to food stimuli moves in the same direction, the small sample size of these sub-groups precludes broader application and a larger scale enquiry should be undertaken.

At the outset of the intervention study with Cohort B, it was the intention to compare an older cohort with excess fat with an older leaner cohort reasoning that the more corpulent group would be more likely to have some degree of insulin resistance. However even those displaying morphological characteristics which would incline them towards insulin resistance; that is a BMI of > 30 kg/m^2 and waist-hip ratio of > 1.0 for men and > 0.8 for women, had fasting and 2 hour post-prandial blood glucose concentrations within the normal range (< 6.1 mmol/l fasting, < 7.8 mmol/l 2 hrs post-prandial). Furthermore the individual Homeostatic Model Assessment (HOMA) scores (Matthews *et al.,* 1985), which is a mathematical model method for detecting insulin resistance, only exceeded 2.0 in 2 individuals (data not shown). There appears to be no reference values for HOMA scores which represent insulin resistance, however scores in excess of 2.00 and 3.99 have been taken as definitive in other studies (Bakari and Onyemelukwe, 2005; Wahreneburg *et al.,* 2005). Plasma insulin concentrations were not significantly different between those with excess fat and lean individuals at any time point and the AUCs for insulin and glucose were not different between the excess fat and lean groups of either gender. Therefore it would seem that glucose homeostasis was still normal in both the lean group and the excess fat group, hence the hypothesis that an altered MCH response may have been observed in the presence of insulin resistance could not be further explored in the current study. The effect of insulin resistance on circulating MCH concentrations therefore requires further investigation.

Contrary to rodent studies, in humans there is little evidence to support a role for circulating MCH in energy homeostasis, therefore it was deemed important to describe associations between RMR and circulating MCH concentrations in young healthy and older individuals. We found no evidence of a relationship between fasted or fed plasma MCH concentrations and RMR in either group. To further explore the relationship between metabolic rate and circulating MCH regression analyses were performed. Results indicate that factors associated with variance in RMR, that is percent fat-free mass, percent fat-mass, fat-free mass (kg) and gender, as well as RMR *per se* do not contribute significantly to the variance in fasted or post-prandial MCH concentrations. These results suggest that circulating MCH cannot be considered a biomarker of resting energy expenditure in humans.

In conclusion, we have demonstrated that circulating MCH can be reliably and quantifiably measured in humans by RIA. Overall circulating MCH concentrations are not overtly reactive and no robust physiological effects of circulating MCH were observed. There does however appear to be some differential regulation in the presence of a combination of gender and adiposity which is variable depending on the population under examination. Hence, in the subjects studied here circulating MCH is not a marker of energy homeostasis, contrary to the suggestion of Gavrila and colleagues (2005). Rather our results suggest that circulating MCH may not have a signalling role in this context although a detailed 24 hour profile of circulating MCH should be established which would lend contextual relevance to the limited body of knowledge regarding circulating MCH in humans to date.

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